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Isolation of RNA From Peripheral Blood Cells: A Validation Study for Molecular Diagnostics by Microarray and Kinetic RT-PCR Assays — Application in Aerospace Medicine

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16. Abstract <p>Gene expression studies in clinical diagnostic settings involve a large number of samples collected at different time points requiring effective methods for collection, transportation, storage, and isolation of RNA to maintain the integrity of expression profiles. Human whole blood is a vital source of RNA for analysis of environmental exposure since blood constituents maintain homeostasis, effect immunity or inflammation, participate in stress signaling, and mediate cellular communication in vascular associated tissues including those of the central nervous system. Isolation strategies using whole blood should recognize the limited quantities of useful RNA that must be protected from the hostile leukocyte ribonucleases, in addition to the abundance of preformed mRNA in reticulocytes, high protein content, and transcriptional activation of cells during sample processing <i>in vitro</i>. This paper presents data showing how the collection, treatment, and storage of collected blood samples can affect subsequent RNA isolation and analysis. It is further demonstrated that total RNA isolated from human whole blood, using a modified and optimized procedure of PAXgene™ Blood RNA reagent kits, performed well in cDNA microarray hybridization and kinetic RT-PCR. Preservation of expression patterns was observed for 96% of the mRNAs after 24 h storage at 4°C by hybridization analysis of 100 mRNA targets. There were no detectable changes in expression levels of 2 housekeeping genes, β-actin and cyclophilin, for up to 10 days storage at 4°C by RT-PCR. This validated protocol was employed for isolation of RNA from blood samples collected in the study of acute ethanol effects on performance and characterization of ethanol inducible biomarkers related to performance impairment. The ultimate goal is to utilize gene expression analysis for aerospace accident investigation and prevention.</p>			
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ISOLATION OF RNA FROM PERIPHERAL BLOOD CELLS: A VALIDATION STUDY FOR MOLECULAR DIAGNOSTICS BY MICROARRAY AND KINETIC RT-PCR ASSAYS — APPLICATION IN AEROSPACE MEDICINE

INTRODUCTION

Extraction of cellular RNA is a critical step in the search for molecular information pertinent to gene expression. Expression profile studies in clinical diagnostic settings often involve samples collected in the field and at different time points, thereby requiring effective methods for collection, transportation, storage, and isolation of RNA to maintain the integrity of expression profiles. Human whole blood is a practical source of RNA for analysis of environmental exposure, performance evaluation, and pathogenesis of diseases because blood constituents maintain homeostasis, effect immunity and inflammation (1), or function as mediators in stress signaling (2-3) and in cellular communication of vascular associated tissues including those of the central nervous system (4-7). However, whole blood is a challenging tissue, since RNA information primarily resides in the small number of circulating blood leukocytes (8). Leukocytes comprise only 0.1% of the blood cellular fraction with approximately 5% of erythrocytes being in different stages of maturation and, therefore, having preformed mRNAs (9). Thus, isolation strategies using whole blood should recognize the limited quantities of useful RNA that must be protected from the hostile leukocyte ribonucleases, in addition to the abundance of preformed mRNA in reticulocytes, high protein content, and transcriptional activation of cells during sample processing *in vitro*. The challenges are how to preserve valid gene expression profiles from sample collection to isolation and how to maximize RNA yields and purity for sensitive downstream analyses. While different uses of nucleic acids have motivated many different ways for mRNA enrichment, the efficiency of these methods has not been determined for expression profiling of human whole blood. This report presents data demonstrating that the collection, treatment, and storage of collected blood samples can affect subsequent RNA isolation and analysis. Further, this report shows that total RNA isolated from human whole blood using the modified procedure of PAXgene™ Blood RNA reagent kit is suitable for gene expression analysis by cDNA microarray and kinetic RT-PCR assays.

MATERIALS AND METHODS

All reagents and supplies were sterile, nucleases free, and of molecular biology grade.

Sample Collection

Human whole blood was obtained from fully informed volunteer donors with the assistance of the Oklahoma Blood Institute (Oklahoma City, OK). Blood specimens were collected using a Safety-Lok™ Blood Collection Set (Becton Dickinson, Franklin Lakes, NJ) directly into PAXgene™ Blood RNA Tubes (Qiagen Inc., Valencia, CA) that contain a proprietary mixture of chemical additives for rupturing blood cells and stabilizing cellular RNA (10). The first 2-mL of blood was collected into an EDTA-coated Vacutainer® Plus tube (Becton Dickinson, Franklin Lakes, NJ) as a discard sample. A total of 50-mL whole blood was collected from each of the 4 donors using quantitative phlebotomy technique. Samples were gently mixed at room temperature (RT) on a hematology/chemistry mixer (Fisher Scientific, Pittsburgh, PA) for at least 2 hours to ensure complete cell lysis and inactivation of ribonucleases prior to isolation of total RNA.

To investigate the effects of storage temperature and aging of samples on RNA recovery, blood samples from the same subject were pooled into a single batch, and then each 18-mL portion of blood solution (≈ 5 mL whole blood + additive solution) was transferred to a sterile 50-mL polypropylene tube (Becton Dickinson, Franklin Lakes, NJ). Sample aliquots were stored at either room temperature (RT) or 4°C for up to 10 days and were processed for RNA isolation at different time intervals (i.e., fresh, 24, 48, 72, 120, and 240 h).

Isolation of Total RNA from Human Whole Blood

Total RNA was isolated according to the PAXgene™ protocol (11) with simple modifications. The nucleic acids were concentrated at $3000 \times g$ in the Eppendorf 5810R (Brinkmann Instruments Inc., Westbury, NY) for 15 min with subsequent removal of the supernatant by aspiration. Each sample had 60 μ L proteinase-K applied to it after the addition of standard volumes of suspension buffer (BR1) and binding buffer (BR2). Protein digestion

was carried out at 55°C for 20 min in a heat block with occasional vortex to ensure mixing. The digested mixture was centrifuged at $10,000 \times g$ for 10 min to collect the supernatant. Following the separation of nucleic acids by affinity column, genomic DNA was digested with RNase-free DNase I directly on the column prior to elution. Contaminating salts and cellular impurities were removed by successive washing, and the RNA was eluted twice in 40 μ L each of elution buffer (BR5). RNA yield and purity were first determined by UV scan (200–400 nm) using Ultrospec 4300 *Pro* (Amersham Biosciences Corp., Piscataway, NJ), with RNA integrity evaluated by electrophoresing 10 μ L of each RNA sample (\approx 0.3–1.0 μ g) from a denaturing gel-loading solution (formaldehyde/formamide/EtBr) on 1.2 % agarose gel in 1 \times MOPS running buffer (pH 7.00). Electrophoresis was performed at 60 V (110 mA) for 1.5 h using the FB650 DC power source (Fisher Biotech, Pittsburgh, PA). Images of the developed gels were acquired by the Typhoon 9210 (Amersham Biosciences Corp., Piscataway, NJ) at a resolution of 100 μ m and 600-V PMT. Selected RNA samples from the refrigerated group were next analyzed by RT-PCR and array hybridization.

Reverse Transcription and Quantitation in Real-Time PCR (RT-QPCR)

To determine the performance of isolated total RNA in RT-PCR, aliquots of total RNA were amplified using specific primers corresponding to regions of human β -actin and cyclophilin genes, according to the protocol previously developed in our laboratory (12). Briefly, RT was performed with 0.6 μ g total RNA and the MMLV reverse transcription system using random primers (Invitrogen, Carlsbad, CA). PlatinumTM *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and Taqman[®] PDAR probe technology (Applied Biosystems, Foster City, CA) were employed in PCR assay of 5 μ L RT products using the Smart Cycler (Cepheid, Sunnyvale, CA) with real-time detection capability. Cyclophilin and β -actin were dually labeled with TET/BHQ-1 (Biosource International, Camarillo, CA) and FAM/TAMRA (Applied Biosystems, Foster City, CA), respectively. Plasmid DNAs with cloned PCR products of β -actin or cyclophilin were used to construct calibration curves for quantitation of the gene transcripts. The plasmids were prepared using pCR[®]II-TOPO[®] vector and TOPO TA cloning technology (Invitrogen, Carlsbad, CA).

Microarray Hybridization

To investigate the effects of storage at 4°C on gene expression profiles, the total RNA isolated from blood samples of one subject was converted to fluorescent-labeled

cDNA using the modified and optimized BD AtlasTM SmartTM Fluorescent Probe Amplification protocol with *random primers* (13). Cy3-labeled cDNAs were generated for RNA isolated fresh and 72 h-aged blood samples, while Cy5-labeled cDNAs were generated for RNA isolated from blood samples after 24 and 240 h storage. The probes were hybridized, via dual labeling scheme to the AtlasTM Glass Trial Microarray (BD Biosciences, Palo Alto, CA) that includes 92 human gene targets and 8 control targets. Images of hybridized arrays were obtained with the Typhoon 9210 Multi-Modal Imager (Amersham Biosciences Corp., Piscataway, NJ) at the set PMT of 750 V. Image analysis was performed in ArrayVisionTM (Imaging Research Inc., St. Catharines, Canada) and SigmaPlotTM (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSIONS

RNA Isolation

Access to RNA requires both cell lysis and inactivation of cellular nucleases during isolation; hence, rigorous denaturing lysis conditions are imperative for obtaining intact RNA (14). Since human whole blood has a high protein content that can reduce extraction efficiency, protocols involving protease digestion should be employed to remove protein contaminants, in addition to increasing RNA yield with greater purity (15). Classical methods using organic solvents can adversely affect the yield and quality of RNA due to the presence of trace phenol and are relatively inefficient for recovery of minute RNA quantities in peripheral blood (16–18). The PAXgeneTM protocol provides reagents for rapid stabilization of cellular RNA during sample collection, along with reagents for removal of hemoglobin and plasma proteins and, therefore, has potential for producing high quality RNA from blood. Preliminary investigation indicated that simple modifications to the PAXgeneTM RNA isolation protocol, enabling 5-mL whole blood to be processed in a single tube, resulted in saving time and reagents, with no significant change in RNA recovery. The yield of RNA from peripheral blood leukocytes acutely depends on the physiologic state of the donor, reflecting the dynamic shift in circulating white blood cell fraction with subsequent wide-ranging variability on RNA constituents and their yields (9,19). Typically, the amount of RNA isolated in this study was 3 – 10 μ g from 5-mL whole blood with a high purity ratio ranging from 1.9 to 2.1, except for RNA from samples that had passed 72 h storage at RT (Table 1). Illustrated in Figure 1 are changes in A_{260} over the elapsed time up to 10 d for samples stored at RT and 4°C. Although the entrapment of nucleic acids in a whitish film of lipids, proteins, or carbohydrates may

Table 1: Yield and Purity of Total RNA Isolated From Human Whole Blood Using PAXgene™ Reagent System

Storage Condition	Yield (SE) ($\mu\text{g}/80\ \mu\text{L}$)	Purity Ratio (SE)
Fresh	7.44 (2.01)	2.18 (0.12)
RT, 24 hr	6.02 (1.74)	2.17 (0.06)
RT, 48 hr	6.38 (0.96)	2.19 (0.04)
RT, 72 hr	3.20 (1.09)	2.16 (0.18)
RT, 120 hr	3.28 (0.52)	1.77 (0.10)
RT, 240 hr	2.28 (0.25)	1.70 (0.02)
4°C, 24 hr	4.50 (0.51)	2.12 (0.09)
4°C, 48 hr	5.30 (1.96)	2.12 (0.08)
4°C, 72 hr	6.70 (1.02)	1.87 (0.05)
4°C, 120 hr	7.63 (1.41)	1.93 (0.03)
4°C, 240 hr	5.97 (0.40)	1.93 (0.04)

TrisCl (10 mM, pH 7.50) was used for reference blank. Values shown are the average of samples from 3 different subjects. Samples from one subject were not used in analysis due to difficulties encountered in phlebotomy and were considered outliers (SE: standard error; RT: room temperature).

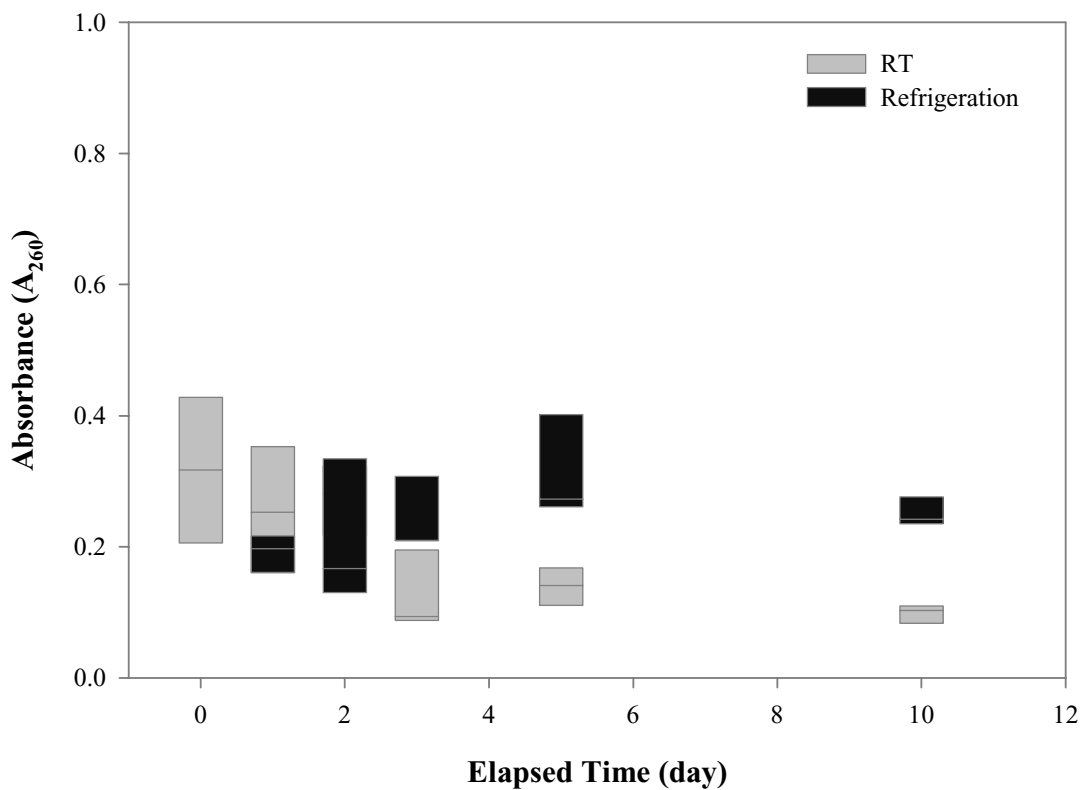


Figure 1: Changes in absorbance A_{260} for RNA isolated from human whole blood stored in PAXgene™ Blood Tubes for up to 10 d at room temperature (RT) and 4°C (Refrigeration). RNAs from freshly collected blood samples were isolated on Day 0 and served as reference for all subsequent comparisons. Lines are median A_{260} values (n = 3).

have contributed to low A_{260} values in some samples, the median A_{260} values for the refrigerated group were not significantly different from that for freshly isolated RNA samples (Friedman; $p = 0.494$). However, there was notable decline in A_{260} within the RT group, starting on the third d of storage, suggesting that peak RNA degradation occurred during this period at RT. Further analysis revealed that average RNA yields were considerably reduced by the 10th d (Dunnett's; $p < 0.05$) and significantly different from average yields obtained from samples stored at 4°C (Bonferroni; $p = 0.006$). Accordingly, blood samples could be maintained for up to 72 h at RT in PAXgeneTM reagents with negligible RNA loss; refrigeration delayed RNA breakdown for a much longer time. To be consistent with such effects, even though contrary to manufacturer's information, it is therefore

prudent to keep blood samples at 4°C if RNA isolation is not promptly performed following sample collection in the PAXgeneTM system of preservatives.

Following gel electrophoresis and depending on the tissue types, total RNA isolated from human tissues should produce a diffuse smear between 0.5 and 12 kb, with 2 bright 28S and 18S rRNA bands at approximately 4.5 and 1.9 kb, respectively. The ratio of the intensities of 28S to 18S rRNA bands should be $\approx 2:1$ (11,14,20). Displayed in Figure 2 is a typical image of molecular weight distribution on 1.2% agarose gel for RNA purified using the PAXgeneTM system protocol. In examining electrophoresis data, the RNA samples exhibited banding patterns characteristic of RNA isolated from human blood. The 2 rRNA bands were within the expected ratio, no extra fragments were observed, and there were no signs

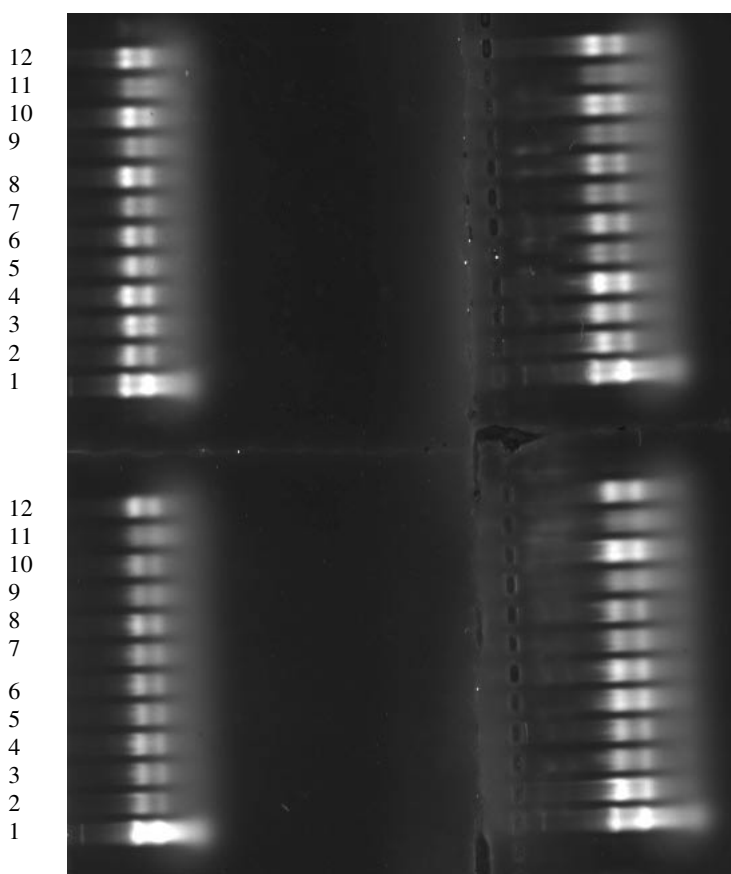


Figure 2: The scanned image of 1.2% agarose gel showing typical banding patterns of total RNA from 4 human blood samples stored in PAXgeneTM Blood Tubes for up to 10 d at room temperature (RT) and 4°C (Refrigeration). (1) 18S & 28S rRNA markers; (2) fresh blood RNA; (3, 5, 7, 9, and 11) RT-stored samples after 24, 48, 72, 120, and 240 h, respectively; (4, 6, 8, 10, and 12) 4°C -stored samples after 24, 48, 72, 120, and 240 h. Degraded RNA samples were observed after 72 h at RT storage (7, 9, and 11).

of genomic DNA contamination. As a result, it was judged that most samples were indeed composed of intact RNA and appeared to be comparable in quality, especially for samples stored at 4°C.

RT-PCR

The isolated RNA was tested by RT-PCR using primer pairs designed to amplify a segment of the β -actin or cyclophilin gene. Both β -actin and cyclophilin genes encode proteins that are essential for basal cellular activities and, therefore, are constitutively expressed in most cells (21-23). Amplification was easily accomplished with 0.6 μ g total RNA isolated from fresh and stored human blood. Concentrations of β -actin (number of actin molecules) at cycle threshold (C_t) were determined from the external calibration curve with log concentration values varied from 6.94 to 6.88, respectively, for fresh and 10-d aged

blood samples (Figure 3). The β -actin concentration ratio was 0.87, which was slightly biased toward fresh blood sample, though not considered significant. Cyclophilin in the same RNA samples produced similar results, with inconsequential changes in C_t and log concentration values after 10 d storage. The resultant cyclophilin concentration ratio was 0.91, suggesting modest deviation in the number of cyclophilin mRNA transcripts that were isolated from fresh and stored blood samples. Although analysis by amplification is dependent on the relative abundance and the stability of the RNA molecule (24), nevertheless, the RT-PCR data, collectively with the electrophoresis and the spectrophotometric results, constitute an indication of negligible damage to total RNA during the 10 d storage at 4°C in PAXgene™ reagent system.

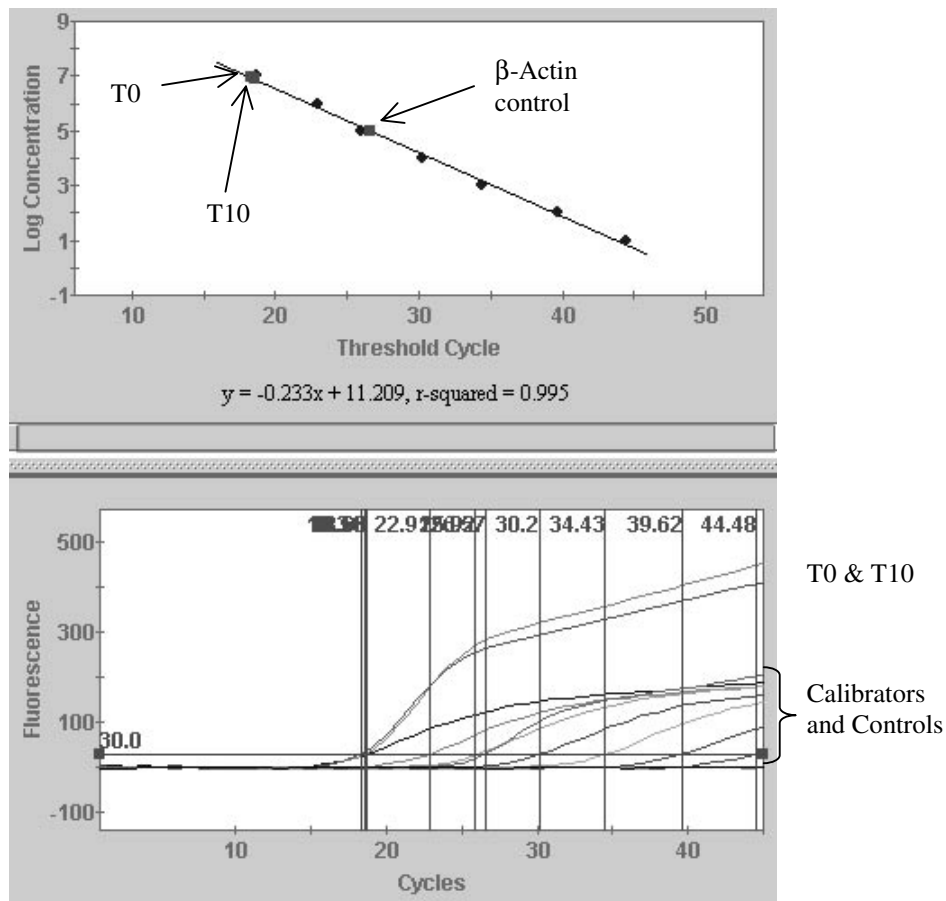


Figure 3: Concentrations of β -actin at cycle threshold (C_t) were determined from the external calibration curve, with log concentration values varying from 6.94 to 6.88, respectively, for fresh (T0) and 10 d-aged blood samples (T10). β -Actin concentration ratio (T10:T0) was 0.87, and the data converged to equivalent points on the calibration curve.

Microarray Hybridization

The Ratio-Average Intensity (RI) plot for data points representing signals produced from intact RNAs on the tested cDNA microarray is illustrated in Figure 4. The intensity ratios for all array elements were based on volume intensity values, which had been background-subtracted, Cy3/Cy5-normalized, and artifact-removed using the analytical algorithm in ArrayVision™. Further normalization based on average intensity for blank elements from the respective arrays allowed pair-wise comparison among the hybridized samples. The derived intensity ratios entailed relative expression levels and were plotted in SigmaPlot™ as a function of log-transformed mean intensity for each gene spot (25-26). Here, log intensity ratios for hybridized cDNA probes produced by 24-, 72-, and 240-h refrigerated blood samples (i.e., T1, T3, and

T10, respectively) were established with a sample from freshly collected blood (T0) as a common reference. The convergence of data points to a tight line corresponding to intensity ratios of $\approx 1:1$ indicates that signal intensities were essentially equivalent for these mRNAs, particularly data points representing extracted RNA from T1 blood sample. However, four genes (M65062, S62045, L34339, D13510) in the T1 and T10 samples deviated negatively from the reference sample as their log intensity ratios ranged from -0.973 to -1.539. Yet, T0 and T3 samples performed similarly in hybridization. This inconsistency in the observed data could reflect the fact that aging of samples was not the only factor that could contribute to the differential detection of these gene elements. It has been recognized that the Smart™ Fluorescent Probe Amplification using *random primers* maintains

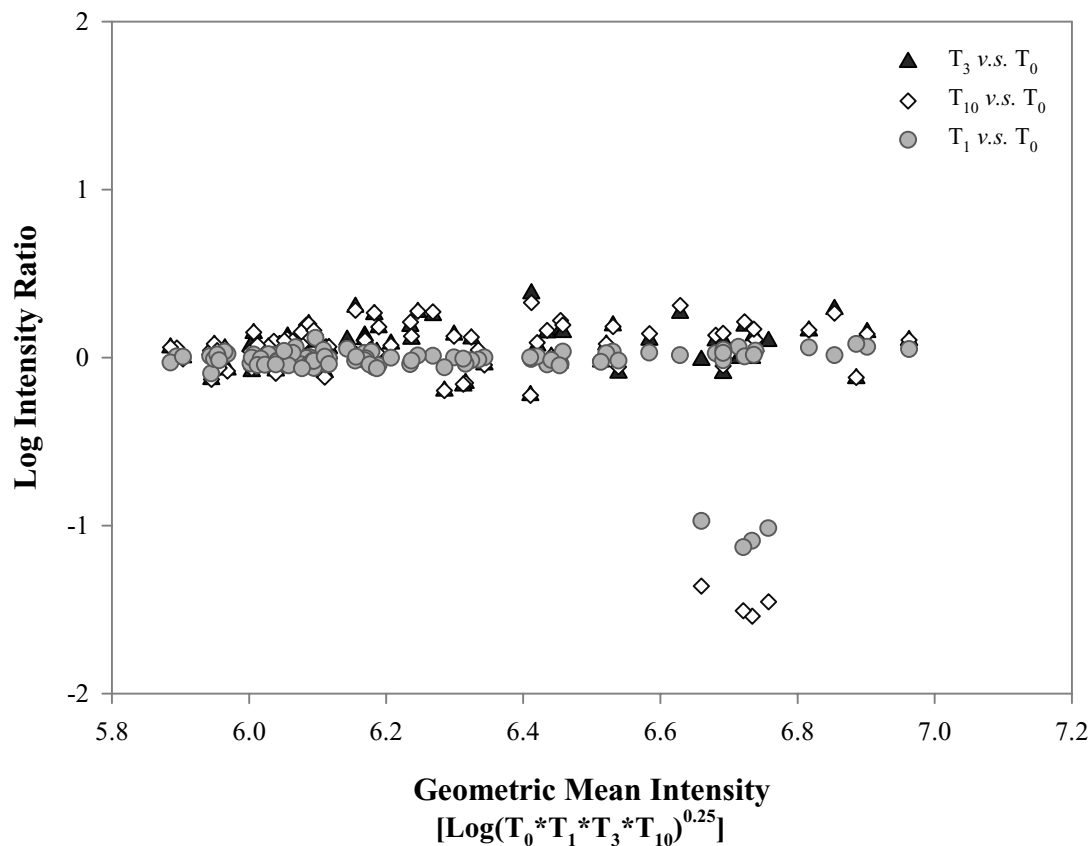


Figure 4: Reproducibility of mRNA profile by microarray hybridization analysis of selected gene targets using Atlas™ Glass Trial Microarrays. The data points for 96 array targets were correlated for all samples tested. cDNA probes were generated for RNA isolated from fresh (T0) and 72 h-stored blood (T3) samples by labeling with Cy3, while Cy5 was used to label cDNAs from 24 h (T1) and 240 h (T10) blood samples. Cy3- and Cy5-labeled probes were hybridized together on the same array. The optimized SMART™ Fluorescent Probe Amplification Protocol was employed using *random primers*.

representation of relative gene expression but can also introduce a slight ratio bias in hybridization signals (27-29). Alternatively, as was discussed in the study conducted in this laboratory (13), faithful replication of gene expression profiles was achieved with *gene specific primers* used in the probe synthesis procedure of the Smart™ protocol. Whereas disparity in hybridized signals remains to be verified by quantitative RT-PCR, preliminary analysis by Bonferroni procedure revealed insignificant differences ($p > 0.05$) between fresh and refrigerated blood samples at all time points. Thus, in spite of the fact that 4 % of the array elements were anti-correlated in a few instances of hybridization, the results from this study demonstrate acceptable reproducibility of the expression profiles using the PAXgene™ reagent systems and the described methods of sample collection and preservation.

SUMMARY

Human whole blood is an essential source of nucleic acids for clinical investigation, since it is readily accessible and rich in molecular information. However, blood is a challenging tissue for RNA isolation and the establishment of a suitable method for collection of experimental samples. All of these procedural steps need to be carefully verified to ensure validity of the experimental data when using blood as a sample source. We evaluated a simple modification of the established protocol that provides a convenient and reliable method for the purification of RNA from fresh human whole blood. A real-time RT-PCR assay was employed to determine RNA quality after isolation, along with microarray hybridization to assess the maintenance of gene expression patterns. The isolated total RNA using PAXgene™ system of reagents was of high purity and demonstrated excellent performance in RT-PCR and hybridization analyses. This report validated the use of PAXgene™ blood collection tubes and RNA isolation reagents for purification of RNA from limited amounts of fresh and stored whole blood collected in human performance studies, specifically in the examination of acute alcohol effects on performance. It is anticipated that this methodology will ultimately enable the characterization of molecular markers related to performance impairment for application in aerospace accident investigation and prevention.

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